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THE KUMAMOTO OYSTER *CRASSOSTREA SIKAMEA* IS NEITHER RARE NOR THREATENED BY HYBRIDIZATION IN THE NORTHERN ARIAKE SEA, JAPAN

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ABSTRACT The status of the Kumamoto oyster *Crassostrea sikamea* in its native Japan is uncertain because of a lack of information about its abundance and distribution and a suggestion that *C. sikamea* and the Pacific oyster *C. gigas* hybridize in the northern Ariake Sea. Furthermore, broodstock populations on the United States Pacific coast have been hybridized with *C. gigas* in the past and may suffer inbreeding depression from multiple generations of hatchery-propagation. As a result, Japanese conservationists and United States oyster growers share an interest in the status of this species in the wild. We collected wild oysters from three sites in Saga Prefecture located in the northern portion of the Ariake Sea, Kyushu, Japan, in September 2006 and used molecular methods (species-specific PCR of the mitochondrial COI gene and PCR-RFLP of the nuclear ribosomal ITS1 gene) to assign 628 sampled oysters to one of three species found in this region. *C. sikamea* proved to be the dominant organism on artificial hard substrates, comprising 91% of the oysters sampled and typed. Many individuals confirmed as *C. sikamea* by diagnostic DNA markers had *C. gigas*-like phenotypes, such as striped shells. *Crassostrea ariakensis* was present (8% of typed oysters) but only at the lowest intertidal levels, and *C. gigas* was rare (1%) at these sites. We found no evidence of hybridization between any of the species and were unable to repeat a previous study, which suggested hybridization between *C. sikamea* and *C. gigas* based on sharing of a calmodulin allele. We conducted gamete compatibility tests among all combinations of Japanese (Ariake Sea) and United States *C. sikamea* and *C. gigas* broodstocks and found strong one-way gamete incompatibility (male *C. sikamea* × female *C. gigas*) between species of Japanese stocks, supporting the molecular diagnosis of *C. sikamea*. However, this one-way incompatibility was less evident in United States stocks, indicating lower barriers to potential hybridization in commercially cultured stocks.

KEY WORDS: *Crassostrea gigas*, *C. sikamea*, *C. ariakensis*, hybridization, conservation, aquaculture, calmodulin, ITS1, COI

INTRODUCTION

The taxonomic status of the Kumamoto oyster *Crassostrea sikamea* (Amemiya 1928) has been the subject of debate over the years. Described as a variety of the Pacific oyster *C. gigas* (Thunberg, 1793) by Amemiya (1928), it was subsequently elevated to full species status by Ahmed (1975). Differences between the two species have been discussed by several authors (Banks et al. 1993, Banks et al. 1994, Hedgecock & Robinson 1992, Imai & Sakai 1961, Numachi 1978, Robinson 1992). Briefly, compared with *C. gigas*, *C. sikamea* is characterized by slower growth, smaller size, a more deeply cupped left valve, and a highly wrinkled or ridged shell. In addition, *C. sikamea* reportedly produces mature eggs in early winter in its native range and in late summer through early winter in the US Pacific Northwest, whereas *C. gigas* has ripe gonads in late spring and early summer in its native and naturalized US habitats (Numachi 1978, Robinson 1992). Further, the eggs of *C. sikamea* are smaller on average than those of *C. gigas* (Numachi 1978, average diameter 27 *versus* 32 μm, respectively, C. J. Langdon, unpublished data). Perhaps, the most important biological evidence supporting the species status of *C. sikamea* is the one-way gametic incompatibility barrier separating it from *C. gigas*. *Crassostrea sikamea* sperm are reportedly incapable of fertilizing *C. gigas* eggs (Banks et al. 1994, Numachi 1978), but the reciprocal cross is fully fertile with eggs from allopatric *C. gigas* and requires concentrated sperm suspensions with eggs from sympatric *C. gigas* (Numachi 1978). Reproductive isolation between *C. sikamea* and *C. gigas* thus appears to be reinforced by natural selection in the Ariake Sea where the species co-occur (Noor 1999).

The most reliable and convenient means of distinguishing between these two oyster species, on the other hand, are biochemical and molecular genetic markers. Variation in allozymes, mitochondrial DNA (mtDNA), and nuclear DNA have all been used as species-specific genetic markers providing unambiguous species diagnosis (Banks et al. 1993, Banks et al. 1994, Buroker et al. 1979, Cordes et al. 2005). Recently, two different research groups have developed species-specific markers for a nontranscribed segment of the multicopy nuclear ribosomal gene (ITS-1, Cordes et al. in review) and a mitochondrial gene (COI, Wang & Guo 2008). The specificity of these markers is further supported by a recent phylogenetic analysis of these two sequences (Reece et al. 2008).

There is considerable uncertainty about the ecological status of *C. sikamea* in Japan. Several attempts by the US oyster culture industry to locate and import *C. sikamea* breeding stock in the early 1990s failed, stoking fears that *C. sikamea* was

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extremely rare or possibly even extinct in its native habitat (Banks et al. 1994, Hedgecock et al. 1999). The Kumamoto Prefecture currently lists *C. sikamea* as an endangered species (http://www.pref.kumamoto.jp/eco/red-list/), although its conservation status is complicated by a lack of information on its distribution and taxonomic confusion with *C. gigas*. Nearby Saga Prefecture lists Suminoe-gaki (*C. ariakensis* [Fujita, 1913] as threatened, and Sikame-gaki as common, http://www.pref.saga.lg.jp/at-contents/kankyo/kankyo/env/nature/index.html), but in this region Sikame-gaki refers to both *C. sikamea* and *C. gigas*, and local fishermen often do not appreciate the distinction. Although Buroker et al. (1979) found *C. sikamea* in their survey of Japanese stocks, Ozaki and Fujio (1985) found only specimens with *C. gigas* allozyme profiles. Hedgecock et al. (1999) examined oysters from 13 sites within the Ariake Sea and, despite intentionally biasing sampling towards oysters with *C. sikamea*-like shell morphology, found that only 20.7% of collected animals were *C. sikamea* based on subsequent genetic testing. In 2004, two of the authors of this report (C. J. Langdon and J. P. Davis) collected over 200 oysters with *C. sikamea*-like morphology from sites in the eastern and southeastern Ariake Sea, which were subsequently identified as *C. gigas*, using molecular markers (M. D. Camara and K. S. Reece, unpublished data).

In contrast, Usuki (2002) reported that *C. sikamea* was common on the northwest and west sides of Ariake Bay based on the diagnostic mitochondrial 16S rDNA marker (Banks et al. 1993). Usuki (2002) further suggested that *C. sikamea* hybridizes with *C. gigas* based on allele-sharing at a putatively, species-diagnostic calmodulin (CaM) genetic marker, which he amplified using the EPIC-PCR primers designed by Côrte Real et al. (1994) for the mussel *Mytilus edulis*. Oysters assigned to *C. gigas* by the 16S rDNA marker were homozygous for one allele, *g*, at the CaM marker, whereas oysters assigned to *C. sikamea* using 16S rDNA had both this allele *g* and an allele, *s*, not found in *C. gigas*. Under the assumption that "true" *C. sikamea* would have only the species-specific *s* allele, Usuki (2002) took the presence of the *g* allele in *C. sikamea* as evidence for hybridization between *C. gigas* and *C. sikamea*. Given the one-way gametic incompatibility between the two species, however, Usuki (2002) expected nonrandom-mating and non-Hardy-Weinberg proportions of the three CaM genotypes *gg*, *gs*, and *ss* in *C. sikamea* but found instead random mating genotypic proportions at two sites (Usuki's Table 9 and Figure 48). The frequency of the *g* allele in *C. sikamea*, which ranged from 0.44–0.62, suggested extensive hybridization, under the assumption that "true" *C. sikamea* would not have this allele. Usuki's (2002) report is at odds with previous evidence that *C. ariakensis*, *C. gigas*, and *C. sikamea* are distinct, sympatric species that are patchily distributed in the Ariake Sea (Banks et al. 1994, Hedgecock et al. 1999, Hedgecock & Robinson 1992, Numachi 1978). Usuki (2002) provided no evidence in support of the key assumption that CaM is a species diagnostic marker, making possible the alternative hypothesis that the CaM *g* allele is simply shared by these two closely related species.

Crassostrea sikamea was first imported to the US in 1947, and by 1953, a total of 3,181 cases of putative *C. sikamea* seed had been planted in Washington, OR, CA, and Hawaii in both experimental and commercial settings (Woelke 1955). In the early 1990s, oyster growers in the US Pacific Northwest were reporting that hatchery-produced *C. sikamea* showed growth rates and morphologies more typical of *C. gigas*, and contamination and/or hybridization was suspected (Hedgecock & Robinson 1992). Subsequent research into diagnostic molecular markers enabled screening of commercial broodstocks and elimination of contaminants (Banks et al. 1993, Hedgecock et al. 1993). Surveys of commercial stocks also provided evidence that at least one hatchery broodstock had a small effective population size likely to promote loss of genetic diversity, inbreeding, and inbreeding depression (Hedgecock et al. 1993). Under the assumption that fresh breeding stock from Japan was unavailable, Hedgecock and Robinson (1992) recommended screening of commercial broodstock using phenotypic and genetic criteria and the establishment of pedigreed, well-managed populations to conserve genetic diversity. Adherence to these recommendations seems to have lapsed, however, as complaints about seed supply (i.e., larval and juvenile survival), size (i.e., growth), and appearance of *C. sikamea* in US markets have recently resurfaced (S. Cudd, B. Eudeline, pers. comm.; J. P. Davis, pers. obs.). Oysters carrying diagnostic DNA markers for *C. sikamea* but resembling *C. gigas* in morphology have raised new questions about the status of US Kumamoto broodstock (J. P. Davis and D. Hedgecock, unpublished data).

Evidence that *C. sikamea* is not extinct in Japan (Hedgecock et al. 1999) provides hope that natural populations can be conserved and that novel germplasm can be obtained to revitalize American breeding stocks. In this paper, we present the results of an expedition to Kyushu, Japan, in the fall of 2006 to determine whether *C. sikamea* can be found and, if so, to collect samples for further genetic study and breeding. A description of the broodstock importation will be published elsewhere. Here, we focus on molecular species diagnosis, inferences about the abundance and distribution of oyster species in the northern Ariake Sea, and gamete incompatibility between sympatric and allopatric populations of the Kumamoto and Pacific oysters, *C. sikamea* and *C. gigas*.

MATERIALS AND METHODS

Field Sampling

On September 24, 2006, we collected 48 individual oysters at several tidal heights from three sites in Saga Prefecture, Japan (Fig. 1; Table 1): the mouths of the Rokkaku River at Suminoe Bridge, the Hama River, and the Kashima River. At all three sites, concrete structures such as boat ramps, piers, or sea walls projecting above the surface of the fine, muddy bottom, were heavily encrusted with oysters. One of three samples from the Rokkaku River site targeted large, flat, round, and smooth-shelled oysters, which occurred very close to the muddy bottom and were believed to be *C. ariakensis*. We returned these samples to the laboratory, where we collected fresh tissue samples for immediate species identification (see later), archived tissue samples of adductor muscle in 95% ethanol for later study and took notes as to whether they were in reproductive condition.

After determining the species composition of these preliminary samples, we returned to the Kashima River site at the predicted low tide of +127 cm MLLW at 17:00 on September 26, 2006 and systematically sampled a transect on a vertical concrete sea wall densely populated with oysters. At a point

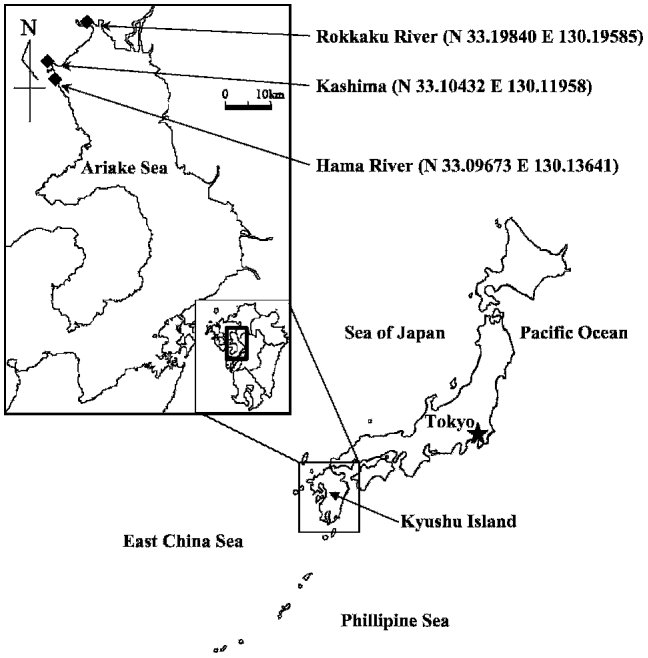

Figure 1. Map of collection sites in the northern Ariake Sea, including latitude and longitude.

2.5 m above the water surface—oysters extended sparsely above this for only another 8 cm—we started a vertical transect, collecting approximately 50 oysters from five, 5 cm-high by ~25 cm-wide horizontal bands, separated from each other by 45 cm of vertical distance. The bottom sample was at the lowest level of oysters growing on the wall, just above the muddy sediment. On the same day, we also collected an additional 81 oysters from the Hama River site that had the outward appearance of *C. gigas* (i.e., striped shells and shallowly cupped valves). We archived adductor muscle samples in 95% ethanol from these field samples and later subdivided and distributed them to three laboratories for molecular analyses (USDA, USC, Tohoku). Finally, on September 28, 2006, we returned to all three sites and collected several hundred live oysters per site for return to the United States as breeding stock. These animals were returned to the quarantine facility at the Hatfield Marine Science Center (HMSC), Newport, OR, USA.

DNA Extraction

In Japan, we used a simple and quick DNA extraction method. Small (~1 mm^2) pieces of fresh mantle tissue were incubated in 150 μL of distilled water, containing 10% Chelex resin (Bio-Rad, Hercules, CA), at 65°C for 35 min and then held at 99.9°C for another 35 min. The Chelex slurry was then mixed for 30–60 s on a Vortex machine and centrifuged at 8,000 rpm for 5 min. The supernatant was used directly as template for PCR.

For ethanol-preserved samples, we used different DNA extraction techniques in different laboratories. In the United States, DNA was extracted from all vertical transect samples in the USDA Shellfish Genetics laboratory, HMSC, Newport, OR, using the Dneasy-96 Tissue Kit (Qiagen, Santa Clara, CA, USA) according to manufacturer's instructions. DNA concentrations were quantified using Pico Green (Molecular Probes, Eugene, OR) and normalized to 5 ng/μL, using a BioMek FX liquid handling system (Beckman-Coulter, Fullerton, CA, USA). The normalized DNA samples, like the tissue samples, were then distributed among the three laboratories. In Japan, new DNA was extracted from the 48 preliminary samples collected from the Rokkaku River, Hama River, and Kashima sites on September 24, 2006 and from the 81 *C. gigas*-like oysters collected from the Hama River site on September 26, 2006, using the Nucleo Spin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

Molecular Species Identification

We used two different molecular techniques to identify oyster species and hybrids (Fig. 2). The first was a multiplex-PCR

TABLE 1.

***Crassostrea* samples collected in the northern Ariake Sea.**

Sample #	Date	Site	n	Description
1	September 24	Rokkaku River	48	Mid-intertidal; concrete boat ramp
2	September 24	Rokkaku River	48	High-intertidal; concrete boat ramp
3	September 24	Kashima River	16	Low-intertidal; concrete boat ramp
4	September 24	Kashima River	48	Mid-intertidal; concrete wall
5	September 24	Kashima River	16	High-intertidal; concrete wall
6	September 24	Hama River	48	Mid-intertidal; concrete boat ramp
7	September 24	Hama River	36	Low/midintertidal; surface of mud flat
8	September 24	Hama River	31	Low-intertidal; floating dock
9	September 24	Rokkaku River	48	Low-intertidal; concrete boat ramp; large, round
10	September 26	Hama River	81	*C. gigas*-like morphology
11	September 26	Kashima River	48	Vertical transect 1; +329.5 cm MLLW
12	September 26	Kashima River	48	Vertical transect 2; +279.5 cm MLLW
13	September 26	Kashima River	48	Vertical transect 3; +229.5 cm MLLW
14	September 26	Kashima River	48	Vertical transect 4; +179.5 cm MLLW
15	September 26	Kashima River	48	Vertical transect 5; +129.5 cm MLLW
16	September 28	Rokkaku River	100+	Large oysters; returned to US alive
17	September 28	Hama River	100+	Large oysters; returned to US alive
18	September 28	Kashima River	100+	Large oysters; returned to US alive

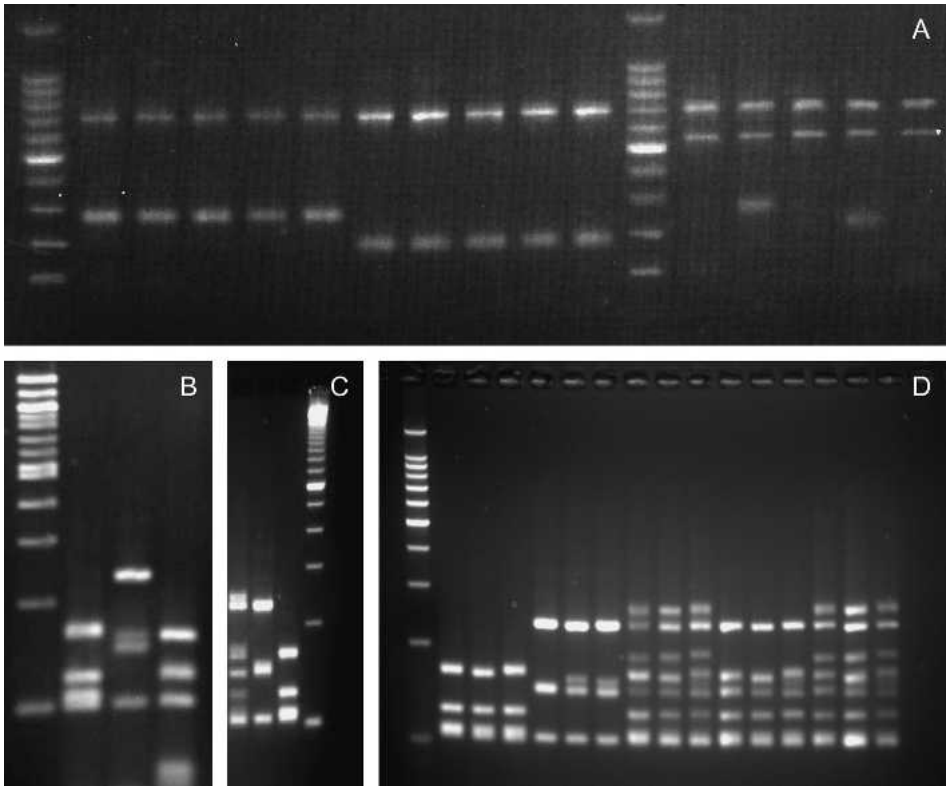

Figure 2. Agarose gel images of species- and hybrid-specific COI and ITS-1 marker genotypes. (A) COI: lanes 1 and 12, size standard (100 bp ladder); lanes 2–6, *C. gigas*; lanes 7–11, *C. ariakensis*; lanes 13–17, *C. sikamea*; (B) ITS-1: lane1, size standard; lane 2, *C. sikamea*; lane 3, *C. gigas*; lane 4, *C. ariakensis*. (C) ITS-1: lane 1, hybrid; lane 2, *C. gigas*; lane 3, *C. sikamea*; lane 4 size standard. (D) ITS-1: lane 1, size standard; Lanes 2–4, *C. sikamea*; lanes 5–7, *C. gigas*; lanes 8–10 *C. gigas* and *C. sikamea* DNA mixed before amplification and digestion; lanes 11–13, *C. gigas* and *C. sikamea* DNA amplified separately and mixed before digestion; lanes 14–16 *C. gigas* and *C. sikamea* DNA amplified separately, mixed, denatured at 95°C, and digested.

assay of mitochondrial cytochrome oxidase I gene (COI) developed by Wang and Guo (2008), which produces species-specific PCR products in a single reaction tube. The second was a PCR-RFLP assay developed by Cordes et al. (in review) for the first internal transcribed spacer (ITS-1) region of the nuclear rRNA-coding gene family. The ITS-1 PCR product was digested with *Hae* III to reveal species-specific RFLP patterns. The PCR products from both assays were visualized on 3.5% agarose gels stained with ethidium bromide.

We observed a hybrid individual at the ITS-1 PCR-RFLP marker (see later). In addition to the expected combination of bands from both parent species, we observed two unexpected bands in this hybrid oyster. These extra bands are heteroduplex DNA molecules formed after denaturation and reannealing of species-specific PCR products (i.e., they are not formed in unheated but *Hae* III-digested mixtures of species-specific PCR products; Fig. 2D).

Amplification and Sequencing of the Calmodulin Intron-3 Region

Following Usuki (2002), we attempted exon-primed, intron-crossing PCR (EPIC-PCR) amplification of calmodulin intron 3, using the *CAD6* and *CAD7* primers developed by Côrte-Real et al. (1994) for the calmodulin-1 (*CaM-1*) gene in *Mytilus edulis*. We also aligned the following mollusc calmodulin sequences to design alternative EPIC-PCR primers: CX726500, abalone (*Haliotis discus*); Gigas-1 (*Crassostrea gigas*) from Dr. Andrew Gracey (Department of Biological Sciences, University of Southern California); AY713401.1, Gigas-3 (*C. gigas*); AY341376.1, Pearl oyster (*Pinctada fucata*); CB416670, Scallop-1, and CB416514, Scallop-2 (*Argopecten irradians*). Alignments of nucleotide and amino acid sequences were done using Clustal X. We targeted conserved exon sequences flanking the intron-3 splice site (amino acid sequence DAD:GNG), and designed new EPIC PCR primers for calmodulin intron-3 (uscCaMF2: 5′-GCCTTTTTGACAAGGA-TGGA-3′ and uscCaMR1: 5′-TGAGGAATTCTGGGAAAT-CG-3′), which were subsequently optimized for Mg^{2+} concentration (1.25 mM) and annealing temperature (57°C). We amplified calmodulin intron 3 in 50 μL PCR reactions from the DNA of six individuals of *C. gigas*, *C. sikamea*, and *C. ariakensis*. PCR products were resolved on a 3% agarose gel, excised, and then purified using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). Products were then sequenced in both directions (High-Throughput Genomics Unit, University of Washington), and these calmodulin sequences have been deposited in GenBank (accession numbers, by species and electrophoretic mobility or size, are: Ca Fast, EU276116; Ca Slow, EU276117; Cg Fast, EU276118; Cg Slow, EU276119; Cs Fast, EU276120; Cs Slow, EU276121).

Gamete Compatibility Assays

To study gamete (in)compatibilities within and between Ariake Sea and U.S. cultured stocks, we artificially crossed two stocks of *C. sikamea* and two stocks of *C. gigas* in a full

4 × 4 factorial mating scheme in which one male from each stock was crossed with three females from each of the four stocks. Each cross was replicated on three consecutive days, yielding 9 replicates for most crosses (three crosses had only 6 replicates; see explanation below).

Parent oysters were either from the US west coast or from broodstock recently collected from the Ariake Sea, Japan. American *C. sikamea* originated from Taylor United Inc., Washington, *via* Whiskey Creek Oyster Hatchery, Oregon, whereas the American stock of *C. gigas* originated from selectively bred families (cohort 18 of the Molluscan Broodstock Program, HMSC), which were derived from naturally spawning populations in Willapa and Dabob Bays, WA, and Pipestem Inlet, British Columbia. Japanese *C. sikamea* broodstock were from the Ariake Sea collections described here. Japanese *C. gigas* were first generation (G_1) progeny of oysters collected from two sites in the southern Ariake Sea in 2004: near the mouth of Midori River or from the walls of the harbor adjacent to the Kumamoto Prefectural Fisheries Research Center near Iwa Jima. We first spawned this *C. gigas* broodstock in February 2006, using pair matings among oysters within each collection site. We reared the G_1 generation at HMSC under quarantine conditions for 16 mo, and oysters from randomly chosen G_1 families were conditioned and spawned for this experiment.

We conditioned both *C. gigas* and *C. sikamea* parent oysters for reproduction by feeding them cultured algae for one month before strip-spawning at 20°C and 25°C respectively. At spawning, we removed eggs from female oysters, transferred them to separate 800 mL volumes of seawater and fertilized them by adding sperm. Simultaneously, we set up control cultures of unfertilized eggs to check for sperm contamination and/or self-fertilization by unrecognized hermaphrodites. After 40 min, we rinsed the eggs with filtered seawater on a 25-μm screen and transferred them to 800 mL of 1-μm filtered seawater. We estimated egg concentrations by counting eggs in a known small volume of seawater under a microscope. For each cross, we incubated 40,000 fertilized eggs in 800 mL of 1-μm filtered seawater for 24 h, at 25 ppt salinity and 25°C. After the incubation period, we counted the number of eggs that developed into normal D-larvae. We analyzed these counts using 4 × 4 contingency table analysis of the entire experiment and a 2 × 2 contingency table of only the putatively incompatible crosses between *C. sikamea* males and *C. gigas* females using the FREQ procedure in SAS v. 9.1 (SAS 2004).

Owing to quarantine space limitations, after counting the D-larvae, we pooled the three replicate crosses per male and raised each of the 48 pools in a separate larval tank. After ~2 wk, competent larvae were allowed to set on clean oyster shell and reared in troughs supplied with running seawater and cultured algae. At approximately 10-wk postspawning, 10 juveniles were sampled from 31 surviving pools and genotyped at both the ITS-1 and COI loci, as were all of the parent animals, as described earlier, to confirm that the expected genotypes were produced in each cross.

RESULTS

Field Samples

Most of the animals sampled and subsequently typed at diagnostic mitochondrial (COI) and nuclear (ITS-1) markers (569 of 628) were found to be *C. sikamea* (Fig. 2; Tables 2 and 3). Of the 299 oysters sampled on September 24, 2007 and sacrificed for immediate species diagnosis, we identified 267 as *C. sikamea*, 1 as *C. gigas*, and 31 as *C. ariakensis*. Our preliminary field survey included two morphologically biased samples—one of large, flat, round, and smooth-shelled oysters from the lower intertidal at the Rokkaku River site and the other of oysters collected at the Hama River site for their *C. gigas*-like appearance. The sample of flat, round oysters was almost entirely *C. ariakensis* (13 of 15), but none of those collected for their *C. gigas*-like morphology at the Hama River site was identified as *C. gigas*. Of the 185 samples subsequently confirmed as *C. sikamea* that were inspected for gross signs of reproductive maturity, only four showed signs of gonadal development. The 92 putative *C. sikamea* subsequently used for broodstock in the United States and the 12 individuals used for gamete incompatibility tests were all confirmed as *C. sikamea*. All identifications were concordant at both markers, providing no evidence for natural hybridization among the three species.

Vertical Transect

Our preliminary field sampling gave us the impression that *C. sikamea* and *C. ariakensis* might be differentially distributed vertically in the intertidal zone. This was confirmed by the distribution of the three oysters species in a vertical transect at the Kashima River site (Table 3). Nearly 90% of the oysters collected in this transect (213 of 239) were *C. sikamea*, except at the lowest tidal level (+130 cm) where *C. sikamea* and *C. ariakensis* were roughly equally abundant (24 *vs.* 22, respectively). Of the 239 individuals typed from the vertical transect, only four were *C. gigas*.

Calmodulin Intron-3

Because our survey results differed so markedly from those reported by Usuki (2002), we attempted to replicate his work.

TABLE 2.

***Crassostrea* species abundance in samples.**

Location	Tidal Zone (sample #)	*C. sikamea*	*C. gigas*	*C. ariakensis*
Rokkaku River	High (1)	47	0	1
Rokkaku River	Mid (2)	48	0	0
Rokkaku River	Very low (large, round; 9)	2	0	13
Rokkaku River	Variable (16)	33	0	0
Kashima River	High (5)	16	0	0
Kashima River	Mid (4)	14	0	2
Kashima River	Low/Mid (3)	16	0	15
Kashima River	Variable (18)	24	0	0
Hama River	Mid/High (6)	15	0	0
Hama River	Low (7+8)	28	2	0
Hama River	Variable (*C. gigas*-like; 10)	81	0	0
	Variable (17)	32	0	0
Total		356	2	31

Sample # and approximate tidal height are from Table 1.
Variable tidal height applies to the sample from the Hama River, which comprised oysters collected nonrandomly for their *C. gigas*-like appearance, and to the broodstock samples, 16–18.

TABLE 3.

***Crassostrea* species composition of samples from Kashima Wall transect.**

Tidal datum (+MLLW)	Sample #	*C. sikamea*	*C. gigas*	*C. ariakensis*
329.5	11	48	0	0
279.5	12	47	1	0
229.5	13	46	2	0
179.5	14	48	0	0
129.5	15	24	1	22
Totals		213	4	22

Using the *CAD6* and *CAD7* primers, we were unable to obtain a specific PCR product for the calmodulin gene. At an annealing temperature of 61°C, we obtained either no product or multiple weak bands and smears on agarose gels; at lower annealing temperatures (50°C to 55°C), only multiple weak bands and smears were observed.

We were able to amplify calmodulin sequences, using newly designed calmodulin intron-3 EPIC-PCR primers and to identify at least two bands on agarose gels in all individuals. The most common bands were ~241 and ~298 base pairs (bp) in length for *C. gigas*, ~241 and ~290 bp for *C. sikamea*, and 247 and 293 bp for *C. ariakensis* (Fig. 3; Fig. 4). We also observed minor bands suggesting intraspecific polymorphism (Fig. 3). Few of these minor bands appeared to be shared among species, however. Consensus DNA sequences for the large and small EPIC-PCR products differed in intron length and nucleotide sequence, as well as in the partial nucleotide and amino acid consensus sequences of flanking exons (Fig. 4). Compared with the larger EPIC-PCR product, the smaller product had an isoleucine instead of a valine just upstream of the intron splice site. This amino acid substitution resulted from two nucleotide substitutions that appeared fixed between the 17 sequences for the larger PCR product and 18 sequences for the smaller PCR product. Fixed or at least highly divergent single nucleotide polymorphisms (SNPs) appeared at three other positions in the alignment of the two PCR products (marked with colons in Fig. 4), two of which are synonymous substitutions. Most of the interspecific differences within the two PCR products (marked with asterisks in Fig. 4) appeared to be synonymous nucleotide substitutions.

The *CAD6* primer of Côrte-Real et al. (1994) aligns with a sequence spanning the first exon-intron boundary (Fig. 4). As in *Mytilus edulis*, it has three mismatches with the sequence of the larger PCR product and four mismatches with the smaller product, including a mismatch at the 3′-end of *CAD6*. The sequence of the *CAD7* primer of Côrte-Real et al. (1994, 3′ to 5′ complement of 5′-GTTTGGTTGTGTAAGAGTAAGG-3′), which was designed to match the *CaM-1* intron 3 sequence of *Mytilus edulis*, did not occur anywhere in any of the oyster CaM intron-3 sequences (*cf.* Fig. 4 with Fig. 3 of Côrte-Real et al. 1994).

Gamete Compatibility Tests

Because we strip spawned relatively small parent oysters, we were only able to collect tissue samples and genotype them after fertilization tests were completed. These tests revealed that one of the putative *C. sikamea* females from the American stock was actually a hybrid resulting from the fertilization of a *C. sikamea* egg with *C. gigas* sperm (genotype = hybrid for ITS-1; *C. sikamea* for CO1). Thus, we eliminated all crosses involving this female from statistical analyses of the D-larvae counts yielding six replicates for three of the cross types (Table 4). No fertilization or development occurred in control treatments of eggs without sperm. Genotypes for parents and juveniles from 31 surviving cross pools were those expected in almost all cases. Five progeny from 4 cross pools had genotypes inconsistent with their putative parents. Because most of these progeny were inconsistent with both parents, we attributed them to accidental transfer of larvae among cultures rather than to sperm contamination.

Counts of the number of D-larvae produced by crosses of *C. sikamea* and *C. gigas* broodstock indicated that intra and interspecific crosses gave rise to normal D-larvae (Table 4). The 4×4 contingency table analysis of all crosses indicated that there were statistically significant differences in the percentage of eggs developing to D-larvae among the cross types ($\chi^2 = 579{,}329$, 9 df, $P < 0.0001$). Intraspecific crosses had the highest rates of fertilization and development to D-larvae, averaging 63% for *C. sikamea* and 55% for *C. gigas*. Eggs from *C. sikamea* developed into D-larvae at slightly lower rates, when fertilized with *C. gigas* sperm, averaging 45%, whereas eggs from *C. gigas* fertilized with *C. sikamea* sperm had the poorest rate of fertilization and development, averaging 11%. A 2×2 contingency

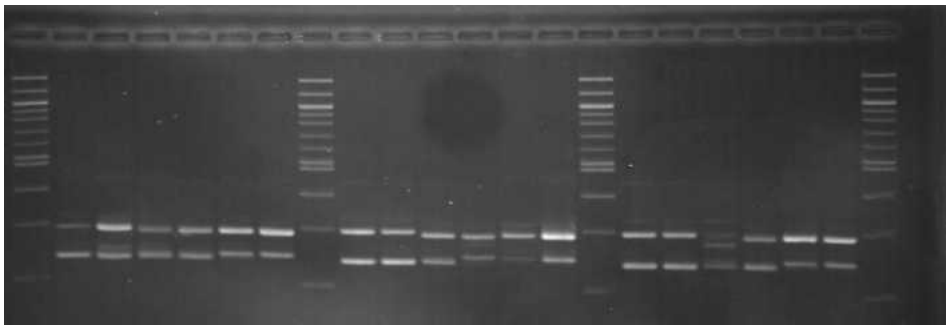

Figure 3. Agarose (3.5%) gel-electrophoresis of CaM intron-3 EPIC PCR products, showing at least 2 bands in every individual (see text for sizes of dominant bands in each species). Size standard in lanes 1, 8, 15, and 22 (100 bp ladder; the 200 bp band is at the bottom); samples between size standards are 2 *C. gigas*, 2 *C. sikamea*, and 2 C. *ariakensis*. CaM PCR products were sequenced for all individuals but the *C. sikamea* in lane 18, which had multiple bands in the size range of the electrophoretically slower PCR product.

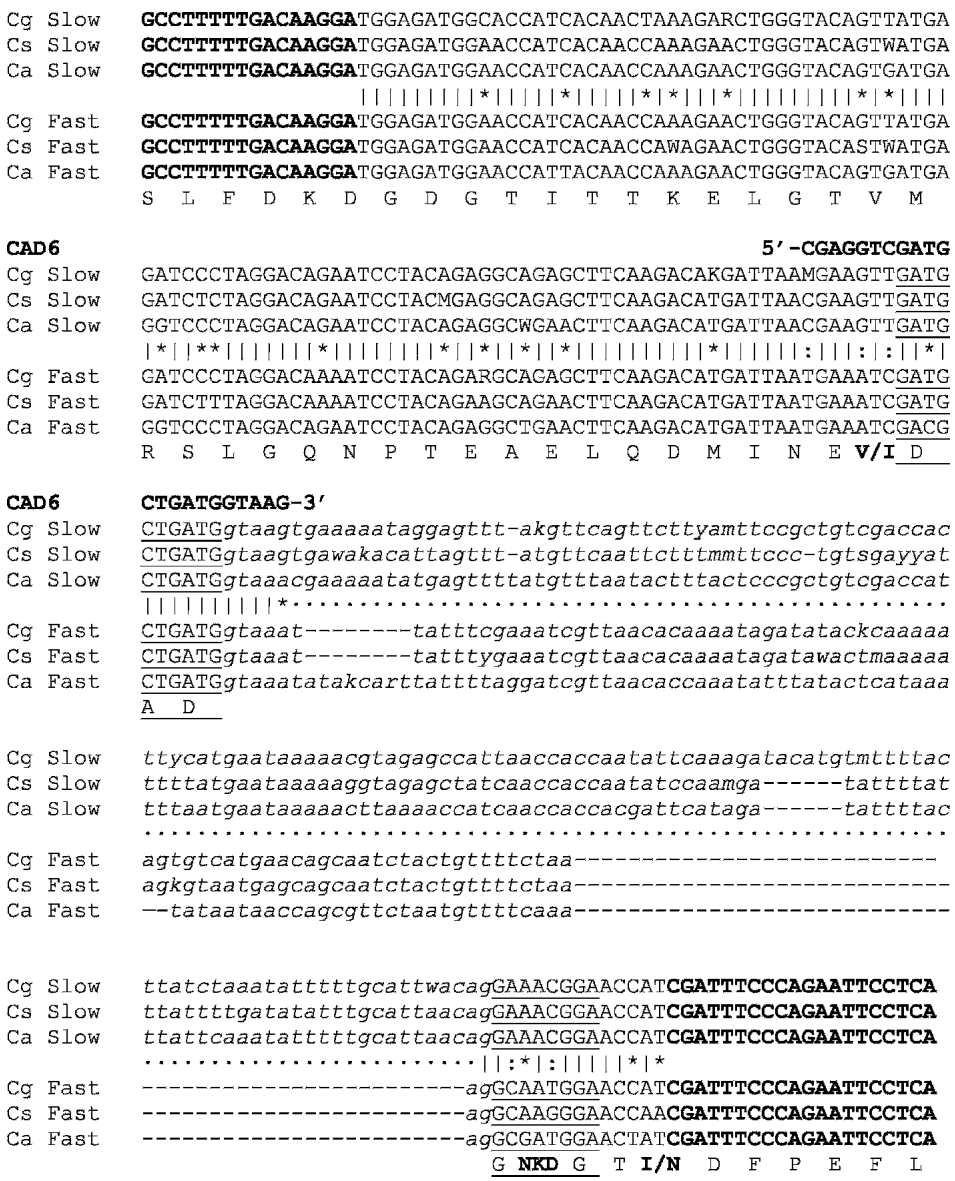

Figure 4. Alignment of consensus nucleotide and amino acid sequences for the large (electrophoretically Slow) and small (electrophoretically Fast) calmodulin EPIC-PCR products obtained from *C. sikamea* (Cs; $n = 5$ for Slow, $n = 6$ for Fast), *C. ariakensis* (Ca; $n = 6$ for both), and *C. gigas* Cg; $n = 6$ for both). Primer sites in bold, including *CAD6* (Côrte-Real et al. 1994). Intron nucleotides are in italicized lower case and aligned only within Slow and Fast sequence blocks. Vertical bars indicate conserved nucleotides; asterisks, one or more substitutions; colons, potentially fixed substitutions between genes. Amino acid replacements indicated in bold. Ambiguous nucleotide codes: K = T/G, M = A/C, R = A/G, S = C/G, W = A/T.

table analysis of only the *C. sikamea* male × *C. gigas* female crosses revealed a significant difference in fertilization rates, depending on the source of the *C. gigas* females ($\chi^2 = 2{,}565$, 1 df, $P < 0.001$). Specifically, a lower proportion of eggs developed into D-larvae from crosses between Japanese or American *C. sikamea* males and Japanese *C. gigas* females (1.1%, 2%, respectively) than developed from crosses between Japanese or American *C. sikamea* males and American *C. gigas* females (13%, 27%, respectively).

DISCUSSION AND CONCLUSIONS

The Kumamoto oyster *Crassostrea sikamea* is the dominant intertidal species on hard substrates in the areas of the northern Ariake Sea that we surveyed, contrary to earlier fears that it might be rare or extinct but in agreement with a 1996 survey (Hedgecock et al. 1999). The species seems in little danger of extinction in this part of its native range. On a vertical sea wall along the Kashima River, we were able to collect ~50 oysters easily from 5 cm × 25 cm areas. This density extrapolates to 4,000 oysters per square meter, 10,000 oysters per linear meter of the 2.5 m-tall intertidal zone dominated by oysters, and many millions of oysters along the sea walls in the vicinity. We estimate that 91% of these oysters are *C. sikamea*.

Cultured *C. sikamea* in the US are deeply cupped with highly ribbed shells that typically lack stripes. Curiously, many of the *C. sikamea* we collected in their native habitat were phenotypically indistinguishable from *C. gigas* and even had some

TABLE 4.

Results of gamete compatibility tests.

Males		Females			
		***C. sikamea* (Japan)**	***C. sikamea* (USA)**	***C. gigas* (Japan)**	***C. gigas* (USA)**
C. sikamea (Japan)	*n*	250,133	258,000	**400**	**48,000**
	P	0.69481	0.71667	**0.00111**	**0.13333**
	SE	0.06088	0.06	0.00111	0.04441
C. sikamea (USA)	*n*	215,067	124,800	**7,200**	**97,600**
	P	0.59741	0.52	**0.02**	**0.27111**
	SE	0.06936	0.10795	0.01167	0.08591
C. gigas (Japan)	*n*	168,133	115,600	160,933	208,800
	P	0.46704	0.48167	0.44704	0.58
	SE	0.06506	0.06436	0.05523	0.06924
C. gigas (USA)	*n*	168,000	95,867	211,467	216,000
	P	0.46667	0.39944	0.58741	0.6
	SE	0.06608	0.05483	0.04514	0.06998

n is the sum of the estimated numbers of D-hinge larvae in replicates of each cross.
Data for crosses of American *C. sikamea* females to all male stocks but the Japanese *C. sikamea* are based on 6 replicate cultures; all other crosses are based on 9 replicate cultures.
P is the mean proportion of starting gametes (40,000 eggs per replicate) surviving to D-hinge.
SE is the standard error of P.
Bold figures denote low fertilization success in "incompatible" *C. sikamea* × *C. gigas* crosses.

characteristics such as striped shells that, at least in the US, are associated with *C. gigas*. Indeed, at first glance, the oysters we found dominating hard substrates at all three sampling sites appeared to be *C. gigas* based on shell morphology and coloration patterns in American stocks. Molecular tests soon revealed that the vast majority was *C. sikamea*. In addition, although we expected based on previous studies (Numachi 1978) that *C. sikamea* would have well-developed gonads in late September for spawning in early winter, we found that almost none of them were in the process of sexual ripening. It will be interesting and necessary to observe the morphology and reproductive cycle of the offspring of these oysters in future generations of cultivation on the US west coast to determine if these unexpected differences have a genetic or environmental basis.

The second most common oyster species, accounting for 8% of the oysters in our samples, is the Suminoe oyster *C. ariakensis*. We found it to be restricted to the lowest levels of the intertidal zone, as reported previously by Usuki (2002). The third most common species, accounting for only 1% of oysters in our samples, is the Pacific oyster *C. gigas*. It seems unlikely that *C. gigas* is abundant enough in the northern Ariake Sea to explain the high frequency of the calmodulin *g* allele in *C. sikamea*—from 0.44–0.62—as the result of interspecific hybridization, as suggested by Usuki (2002). In fact, we found no evidence of interspecific hybridization using diagnostic COI and ITS-1 markers, even though formation of ITS-1 heteroduplex bands in hybrids would have facilitated identification of the most likely interspecific hybrids from the *C. gigas* male × *C. sikamea* female cross (see discussion of gamete compatibility below).

Our attempts to replicate Usuki's (2002) results for CaM intron 3 were unsuccessful. We were unable, first, to obtain a specific EPIC-PCR product for the calmodulin gene following the methods used by Usuki. To investigate this failure further, we designed conserved EPIC-PCR primers to amplify molluscan CaM intron 3, obtained PCR products, and generated oyster-specific calmodulin sequences. We found that, as in *Mytilus edulis* (Côrte-Real et al. 1994) and other molluscs, all three species of Asian cupped oysters examined in this study have at least two calmodulin genes. The two genes differ in the length and sequence of intron 3 and in the partial amino acid and nucleotide sequences of flanking exons (Fig. 3). The *CAD6* primer designed for *M. edulis* by Côrte-Real et al. (1994) and used for oysters by Usuki (2002) matches the larger intron-3 PCR product better than the smaller one, most notably at the 3′ end, suggesting that this PCR product may be homologous with *CaM-1* in *M. edulis* (Côrte-Real et al. 1994). A matching sequence for *CAD7* does not exist in any of the oyster intron-3 sequences we examined, however, which explains our failure to obtain the PCR products reported by Usuki (2002).

The sizes of our two calmodulin intron-3 EPIC-PCR products (~240 bp and ~290 bp) are much smaller than the PCR products (445 bp and 519 bp) reported by Usuki (2002) as alleles of a single polymorphic, calmodulin gene. This discrepancy in intron-3 size is even larger than these numbers suggest. *CAD6* crosses the 5′ exon-intron boundary and *CAD7* is inside of intron 3 whereas our primers start 105 bp upstream of and 14 bp downstream of the intron-3 splice site, respectively (Fig. 4) and should produce much larger amplicons than the *CAD6*/*CAD7* primer pair. Though minor intraspecific polymorphisms are apparent in our gel analysis of calmodulin PCR products (Fig. 3), these polymorphisms for the most part do not appear to be shared among species. Further work may well find that these calmodulin markers, which contained various gap and nucleotide polymorphisms even in our small samples, might serve as new species-diagnostic markers for Ariake Sea oysters. Nevertheless, our failure to confirm Usuki's calmodulin results, especially the failure to find calmodulin sequences matching the *CAD7* primer that he used, casts doubt on the validity of his evidence that *C. sikamea* and *C. gigas* hybridize extensively in the Ariake Sea. Concordant diagnoses of *C. sikamea* using mitochondrial (COI) and nuclear (ITS-1) diagnostic markers, on the other hand, taken together with results from gamete

compatibility tests, affirm that the Kumamoto oyster *C. sikamea* is an abundant biological species in the northern parts of the Ariake Sea.

Gamete compatibility tests supported the identification of oysters that were imported to the United States as *C. sikamea*. Banks et al. (1994) previously reported zero fertilization of *C. gigas* eggs with *C. sikamea* sperm—none of 135 eggs from an American *C. gigas* female were cleaving when examined at 1 h postfertilization with sperm from American *C. sikamea*. We found not complete incompatibility, but rather dramatically reduced fertilization of *C. gigas* eggs by sperm from *C. sikamea* compared with rates of fertilization for intraspecific crosses or the reciprocal hybrid cross of *C. gigas* sperm × *C. sikamea* eggs. Using nine replicated cultures of 40,000 eggs for each cross type, we found 1% to 2% fertilization in crosses of *C. sikamea* sperm with eggs of *C. gigas* from the Kumamoto Prefecture, Japan. Such a low percent fertilization compared with the previously reported zero percent could be explained by the much larger sample size in this study. However, we found much higher rates of fertilization (13% to 27%) in crosses of *C. sikamea* sperm, especially from the American stock, with eggs from US *C. gigas* females, the same cross for which Banks et al. (1994) reported zero fertilization. Whereas the causes of this discrepancy are unknown, the lower gamete compatibility in sympatric *versus* allopatric crosses is nevertheless consistent with reinforcement of reproductive isolation in sympatry (Noor 1999). We observed a significantly higher level of gamete compatibility in *C. sikamea* × *C. gigas* crosses when eggs came from US Pacific oysters originally imported mostly from the Miyagi Prefecture in northern Japan (Mann 1979), than when eggs came from Ariake Sea *C. gigas* females. The allopatric origins of *C. sikamea* and *C. gigas* stocks in the US Pacific Northwest make hatchery-propagated Kumamoto oyster broodstocks all the more vulnerable to hybridization. To what extent this vulnerability of allopatric stocks to hybridization may have been exacerbated by previous hybridization events or domestication is unknown.

The one hybrid female, which was found among the 12 American *C. sikamea* used in our gamete compatibility tests, confirms that American broodstocks of *C. sikamea* still contain hybrids formed by the compatible cross of *C. gigas* sperm with *C. sikamea* eggs. This was a fortuitous event for two reasons. First, this individual and her progeny enabled us to observe the appearance of heteroduplex DNA created in PCR amplification of the ITS-1 diagnostic marker from an interspecific hybrid (Fig. 2C,D). Heteroduplex bands are more easily detected on agarose gels than a simple additive hybrid pattern would be, increasing the likelihood of detecting hybridization in surveys of natural Japanese populations of *C. sikamea*. Second, the fertility of this female in crosses with *C. sikamea* males from US hatchery stocks (73% of fertilized eggs surviving to D-larvae) and *C. gigas* males from US stocks and the Ariake Sea (66% and 63%, respectively) is the first evidence that *C. gigas* × *C. sikamea* hybrids are fertile in backcrosses to either parent. This heightens concern about the consequences of interspecific hybridization either in hatchery propagated stocks or in the wild should it ever occur.

Our expedition, which was aimed at finding and importing live *C. sikamea* to the United States, sampled extensively in only three localities on the northern shore of the Ariake Sea. Further study is needed of *C. sikamea* in Japan to elucidate the species distribution and abundance, its geographical range, ecological requirements, and genetic diversity within and among local populations. More complete information would serve two different but complementary objectives: laying the groundwork for more effective management of the species and its diversity in the Ariake Sea and the conservation of germplasm resources for an important aquacultural species in the United States.

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